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(54) Title: OLIGONUCLEOTIDE CONJUGATION

(57) Abstract

The present invention provides a method for the conjugation of peptide molecules with oligonucleotide molecules by means of accessory molecules attached to the peptide or oligonucleotide molecules performing the Diels Alder reaction. Specifically, either a diene or dienophile moiety is attached to a peptide molecule, with the moiety which is not selected above being attached to an oligonucleotide, this attachement being facilitated by a number of possible ways. The pursuing Diels Alder reaction results in the diene and dienophile groups forming a sic membered cyclohexane ring structure which, due to the attachement of the diene and dienophile with the peptide and oligonucleotide molecules participating molecules in the reaction, serves to form a peptide oligonucleotide hybrid molecule.

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"Oligonucleotide Conjugation"

The present invention relates to a method for the conjugation of peptide molecules with oligonucleotides. More particularly, the invention relates to a means whereby specific accessory molecules attached to the peptide and oligonucleotide molecules can be fused together to form a synthetic 6-membered cyclohexane ring through the induction of the Diels Alder reaction.

Peptide oligonucleotide hybrid molecules (conjugates) are a class of molecular construct which have a potentially wide application in several fields of biotechnology. Although some efforts have been made in this area, there is to date no universally accepted methodology to attach peptide fragments to oligonucleotides. Thus there is scope to develop a generalised and simple method to attach peptides to oligos.

Several attempts have been made to produce hybrid molecules which comprise both peptide and

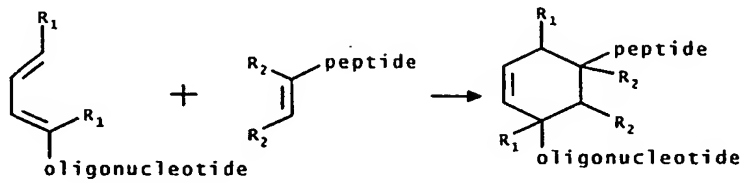
oligonucleotide portions directly by solid phase synthesis. All of these attempts have met with various difficulties. Thus manual peptide synthesis followed by automated oligonucleotide synthesis was accomplished by Haralambidis et al., 1990 using controlled pore glass as a solid phase support and by Juby et al., 1991 using Teflon fibres. Neither of these groups was able to fully automate the process and difficulties were encountered as these supports although suitable for oligonucleotide synthesis are not ideal for peptide synthesis. Truffert et al., 1994 report the fully automated synthesis of conjugates using silica supports including controlled pore glass, but in low yield. Further, in this approach very large excesses of reagents and extended coupling times were necessary in some of the steps. The deprotection conditions reported also led to the peptide portion of the hybrid molecule being produced as an unnatural C-terminal ethanolamide. The approach to the problem described by Basu and Wickstrom, 1995 uses a different solid phase support based on a bifunctional linker attached to a polyethylene glycol-polystyrene. This method, however required the use of specially protected nucleotide monomers and suffered from low overall yields. A further report on the automated synthesis of conjugates suitable for use as primers (Tong et al., 1993) produces the target molecules only in very low yields by a very inefficient route. None of these strategies are ideal for the generation of a library of molecules where a given set of oligonucleotides is specifically combined with a set of peptides to generate a source of

molecular diversity as is used in the increasingly important combinatorial techniques.

The other strategy which has been adopted for the synthesis of oligonucleotide peptide conjugates uses the post synthetic conjugation of separately synthesised and purified oligonucleotide and peptide segments. Linkages between the two segments have been accomplished by a variety of techniques including the formation of a disulphide (see for example Wei et al., 1994) or a thioether (see for example Ede et al., 1994; Harrison and Balasubramanian, 1998). The use of a thiol group to make the linkage in these methods make it difficult to introduce a peptide segment which contains a free cysteine residue in the final conjugate. Similarly, the approach of Bayard et al., 1986 which makes use of the reaction between an amino group and an oligonucleotide aldehyde leads to difficulties in incorporating some amino acids easily into the peptide segment of the molecule and in addition requires the generation of an oligonucleotide aldehyde which is a process liable to damage the nucleotide segment and lead to complex by-products. The elegant template directed ligation of oligonucleotides to peptides described by Bruick et al., 1996 requires the preparation of a complex set of modified oligonucleotides and a peptide as a C-terminal thioacid. Two of the three oligonucleotides required for this method do not appear in the final conjugated product and this method, although ingenious, is not suitable for routine use.

It is an object of the present invention to provide a method for the conjugation of a peptide and an oligonucleotide to form a hybrid molecule, through the use of associated molecules participating in the Diels Alder reaction. The Diels Alder reaction can take place under exceptionally mild conditions, wherein a diene moiety reacts with a dienophile moiety to form a 6-membered cyclohexane ring structure. In the present invention either a diene or dienophile moeity is attached to a peptide component, with the moiety which is not to the peptide, being attached to an oligonucleotide. The proceeding Diels Alder reaction results in the formation of combining of the diene and dienophile, the result being that their associated molecules form a peptide oligonucleotide hybrid molecule.

According to the present invention, there is provided a process for conjugating a peptide with an oligonucleotide, the process comprising the steps of attaching a diene to the peptide and a dienophile to the oligonucleotide, or attaching a diene to the oligonucleotide and a dienophile to the peptide, and reacting the so-formed components by means of the following reaction,



wherein R1 and R1' are electron donating groups and R2 and R2' are electron withdrawing groups or R1 and R1' are electron withdrawing groups and R2 and R2' are electron donating groups.

This reaction is the Diels Alder reaction.

R1 and R1' may be the same or different. R2 and R2' may be the same or different. Where R1 and R1' are electron donating groups, R2 and R2' are electron withdrawing groups and *vice versa*.

Electron donating groups may be chosen, though not limited from the selection consisting of hydrogen, alkyl, cycloalkyl, aryl, S, O, N or heterocyclic structures including these. Electron withdrawing groups may be chosen, though not limited to nitro, nitrile, sulphonic acid, carboxylic acid, aldehyde, carbonyl (C=O-R, where R may be N, O or S, alkyl, cycloalkyl, or aryl), phosphate, sulphone, quaternary ammonium or heterocylic structures containing these. Further, the electron withdrawing groups and/or the electron donating groups may be joined such that they themselves form part of a cyclic structure, as is the case in the present examples, or they may be acyclic.

Preferably all the peptide moieties are attached to only one of either a diene or a dienophile group, and all the oligonucleotide moities are attached to the other group.

Also preferably the diene and dienophile are reacted under conditions suitable for a Diels Alder reaction, wherein the formation of a cyclohexene ring structure provides a means for joining molecules associated therewith, thus forming a peptide oligonucleotide hybrid molecule.

The invention thus provides a new method in which a peptide and oligonucleotide can be conjugated into a hybrid molecule. This method of conjugation facilitates a novel use of the Diels Alder reaction, through the original attachment of the substrates for this reaction to the molecules which are required to be conjugated.

In one embodiment of the present invention, it is proposed to accomplish conjugation by attaching the dienophile to the peptide moiety. This can be accomplished by using an N-malemide group on the peptide (Keller & Rudinger, 1975). This group is stable to the harsh acidic conditions used for peptide cleavage, but is not compatible with the commonly used FMOC synthesis strategy whose repeated base deprotection steps would cleave the group. Thus the maleimido amino acid can be incorporated as the last step in the synthesis of the peptide. This represents no real disadvantage, as either the linkage will be (most simply) at the N terminus, or strategies can be used to specifically deprotect an internal amino group and derivatise this appropriately. The only real problem foreseen in this approach to the peptide derivatisation is in dealing with SH groups, but

strategies to overcome this are also available, as these are usually unmasked only at the very last step of peptide synthesis, prior to their oxidation if this is appropriate.

The diene component of the reaction pair will be preferentially attached to the oligonucleotide portion.

The attachment may be facilitated by a post synthetic strategy wherein an amino functionalised oligonucleotide is reacted with an active ester with the required diene, resulting in the diene attaching to the oligonucleotide.

Alternatively, the diene may be added during the synthesis of the oligonucleotide, through the incorporation of the required functional group using a phosporamidite monomer.

The diene may be attached to the 3' or 5' terminus of the oligonucleotide.

Preferably the diene may be attached by means of a non-nucleoside linker.

Alternatively, the diene may be attached to the nucleoside by means of a pendant arm bearing the diene group being attached at the N4 position of the deoxy cytidine, the 5 position of pyrimidines, the 8 position of purines and the 2 position of the ribose portion of any nucleoside.

The Diels Alder conjugation reaction linking the dienophile associated peptide to the diene associated oligonucleotide should proceed smoothly. Such reactions are especially favourable in highly polar aqueous environments, as is the case when carrying out this reaction between a peptide and an oligonucleotide which are both water soluble.

A conjugated diene could be reacted with a dienophile, especially a dienophile with an attached electron withdrawing group, to facilitate the Diels Alder reaction. With a conjugated diene, a concerted reaction proceeds through a cyclic transition state, forming a cyclohexene ring structure.

The information will be demonstrated in the following examples with reference to the accompanying figures wherein:

Figure 1 illustrates the Diels Alder reaction.

Figure 2 illustrates the post-synthetic attachment of a diene to an oligonucleotide.

Figure 3 illustrates the conjugation of a peptide molecule to an oligonucleotide by means of the present invention.

Figure 4 shows the N-furfuryl deoxycitidine phosphoramidite monomer.

Figure 5 shows a phosphoramidite monomer.

A generalised reaction scheme of the Diels Alder reaction is shown in Figure 1. In this figure, R1 is an electron donating group and R2 is an electron withdrawing group, this is the 'normal' Diels Alder reaction. The present invention could also make use of the 'inverse electron demand' Diels Alder reaction where R1 is an electron withdrawing group and R2 is an electron donating group.

Electron donating groups (R1, R1') may be chosen, though not limited from the selection consisting of H, alkyl, cycloalkyl, aryl, S, O, N or heterocyclic structures including these. Electron withdrawing groups (R2, R2') may be chosen, though not limited to nitro, nitrile, sulphonic acid, carboxylic acid, aldehyde, carbonyl (C=O-R, where R may be N, O or S, alkyl, cycloalkyl, or aryl), phosphate, sulphone, quaternary ammonium or heterocylic structures containing these. Further, R1 and R1' and/or R2 and R2' may be joined such that they themselves form part of a cyclic structure as is the case in the present examples, or they may be acyclic.

Although in the present example, the two electron withdrawing and donating groups are the same, this does not necessarily always have to be the case, as both the diene and dienophile molecules could be asymmetric, giving R1 and R1' in the one case and R2 and R2' in another.

It should be noted that in general terms, the position of the reacting portion on the oligonucleotide could be at the 3' or the 5' terminus or could be attached to a suitably functionalised nucleotide in the sequence. Similarly, the peptide portion could bear its reacting group either at the N terminus or at some side chain in the peptide which bears suitable functionality. In addition, the diene component could be attached to the peptide and the dienophile to the oligonucleotide or vice versa.

The oligonucleotide in the present example is an oligodeoxynucleotide, but in principle, the method could be used in the preparation of oligoribonucleotide peptide conjugates.

More generally, this scheme could be used to form conjugates between oligonucleotides and any other suitable molecules which could be attached to a diene or dienophile. Similarly, it could be used to form conjugates between peptides any other suitable molecules bearing a diene or dienophile.

In the specific examples given below, the diene group has been attached either through the use of a non-nucleoside linker or by using an attachment strategy on the N-4 position of deoxycytidine. However it should be noted that other positions of nucleoside molecules are suitable for derivatisation with pendant arms bearing diene groups. Notable among these are the 5-position of pyrimidines, the 8-position of purines and the 2'-position of the ribose portion of any

nucleoside, since these are well known to cause minimal perturbation to DNA structures.

Examples

It is proposed to accomplish the conjugation by attaching the dienophile to the peptide moiety of the peptide oligonucleotide pair. This is easily accomplished by using an N-maleimide group on the peptide (Keller & Rudinger, 1975). This group is stable to the harsh acid conditions used for peptide cleavage, but is not compatible with the commonly used FMOC synthesis strategy whose repeated base deprotection steps would cleave this group. Thus the maleimido amino acid would be incorporated as the last step in the synthesis of the peptide. This represents no real disadvantage, as either the linkage will be (most simply) at the N terminus or strategies can be used to specifically deprotect an internal amino group and derivatise this appropriately. The only real problem foreseen in this approach to the peptide derivatisation is in dealing with thiol groups, but strategies to overcome this are also available, as these are usually unmasked only as the very last step of peptide synthesis, prior to their oxidation if this is appropriate.

It is proposed that the diene component of the reaction pair be attached to the oligonucleotide portion. It is envisaged that the preparation of both the peptide and oligonucleotide portions should be relatively simple. Figure 2 illustrates, by means of a non-limiting

example the post synthetic attachment of a diene to an oligonucleotide.

In this illustrative case, an oligodeoxynucleotide was prepared by standard techniques using an automated synthesiser and commercially available monomers. In the last step of the synthesis a monomer was used which allows the incorporation of an amino group at the 5' end of the oligonucleotide (1). Purification of the oligonucleotide was by means of a commercial purification cartridge system. This oligonucleotide was then reacted with the active ester furan derivative (2) to produce the diene bearing oligonucleotide component (3).

Figure 3 further shows the conjugation of a peptide molecule with associated dienophile to diene bearing oligonucleotide.

As a simple model peptide, the N-maleoyl derivative of 6-aminohexanoic acid(4) was prepared and reacted with the diene-bearing oligonucleotide derivative(3) to yield the target oligonucleotide peptide conjugate (5). All reactions proceeded smoothly and in high yield at room temperature. The reactions were followed by hplc and the identities of the products confirmed by electrospray mass spectrometry.

In this example, the diene component has been attached to the oligonucleotide by means of a post-synthetic strategy, by first preparing an amino functionalised oligonucleotide and then reacting this with an active

ester bearing the required diene moiety. In further examples, this methodology for attaching the diene (or dienophile) to the oligonucleotide has been shortened by the incorporation of the required functional group using a suitable phosphoramidite monomer, eliminating the need for this extra post-synthetic step in the procedure.

By way of illustration of this, two phosphoramidite monomers (6) and (7) were prepared by means of a bisulphite catalysed transamination reaction (Tesler et.al., 1989) and incorporated into oligonucleotides using standard techniques on an automated synthesiser. These molecules are graphically represented in Figures 4 and 5.

Use of these monomers did not require the modification of the synthetic cycle and they behaved in all respects as standard unmodified monomers. Coupling efficiencies of the modified monomers was comparable to that of the unmodified compounds. These modified monomers can be introduced into the sequence either terminally or internally.

The utility of the oligonucleotides so produced in the construction of conjugates was verified by the synthesis of model peptide oligonucleotide hybrid molecules as described above and also in the construction of oligonucleotide enzyme hybrid molecules which have utility in the non-radioactive detection of nucleic acid sequences. To this latter end, an oligonucleotide was efficiently coupled with the enzyme

alkaline phosphatase, using commercially available maleimide-modified enzyme.

The peptide component design is compatible with standard peptide synthetic strategies and the reactive group survives peptide workup conditions.

The Diels Alder conjugation reaction linking the peptide to the oligonucleotide should proceed smoothly. It has been shown that the reaction takes place under aqueous conditions at room temperature. There is evidence in the literature that such reactions are especially favourable in highly polar aqueous environments as is the case when carrying out this reaction between a peptide and an oligonucleotide which are both water soluble. There is further evidence that because of the very mild and specific nature of the Diels Alder reaction unwanted side reactions are minimal.

Preparation of 6-maleimidocaproic acid (4)

The required compound was obtained by the method of Keller & Rudinger, 1975.

Preparation of furan active ester (2)

Furfurylamine (1.94g, 20mmol) and adipic acid monomethyl ester (3.2g, 20mmol) were dissolved in dry dichloromethane (40 ml) under an argon atmosphere and cooled to 0°C. A solution of N,N'-dicyclohexylcarbodiimide (4.12g, 20mmol) in

dichloromethane (40 ml) was rapidly added to the stirred solution and the reaction mixture left to stir overnight at room temperature. The reaction mixture was then cooled on ice and the precipitate filtered off. The filtrate was evaporated and the resulting residue purified by flash column chromatography on silica gel, eluting with ethyl acetate : pentane (1:1 vol/vol) to yield the amido ester as a low melting solid (3.60g, 76%).

The amido ester (2.39g, 10mmol) was added to 1M sodium hydroxide solution in methanol: water (2:1 vol/vol) and allowed to react for 1.75 hours, after which time tlc analysis showed that hydrolysis was complete. Dowex ion exchange resin (H+ form) was added to neutralise the reaction. The ion exchange resin was filtered off and washed with a small volume of methanol: water. The filtrate was evaporated to dryness and co-evaporated with toluene (3 x 30ml). This solid residue was dissolved in dry N,N-dimethylformamide (30 ml). N,N'-dicyclohexylcarbodiimide (2.06g, 10mmol) and N-hydroxysuccinimide (1.15g, 10 mmol) were added. After stirring at room temperature for 2 hours, the reaction mixture was cooled on ice and the precipitate filtered off. The filtrate was evaporated under reduced pressure to yield a semisolid residue which after aqueous workup was purified by flash column chromatography using a gradient of ethyl acetate in dichloromethane (1:4 to 1:1). This yielded the desired product (2) as a solid mpt 82-83°C. Tlc rf (dichloromethane: ethyl acetate 2:1) 0.33

$^{1}H$ nmr: 200 MHz (CDCl3) 7.35 (m, 1H); 6.32 (m, 1H); 6.23 (m, 1H); 6.03 (br s, 1H); 4.43 (d, 2H); 2.84 (s, 4H); 2.64 (m, 2H); 2.26 (m, 2H); 1.80 (m, 4H)
$^{13}C$ nmr: 50 MHz (CDCl3) 172.06; 169.25; 168.35; 151.36; 141.99; 110.31; 107.20; 36.03; 35.48; 30.52; 25.47; 24.45; 23.87

Preparation of oligonucleotide (1)

The oligodeoxynucleotide was prepared on a 0.2 µmol scale on an ABI 381A synthesiser in trityl-on mode using standard protocols for cyanoethyl phosphoramidite chemistry. 5'-Amino modifier C 6 (Glen Research Corporation, Sterling, VA, USA) was incorporated at the 5' end of the oligonucleotide. The oligonucleotide was deprotected and purified using the PolyPak cartridge system (Glen Research Corporation, Sterling, VA, USA) according to the manufacturer's instructions.

The sequence synthesised was (5') C6 aminolink GTATCACGAT (3'). Coupling yields during synthesis (as monitored by the release of dimethoxy trityl cation) were > 99.5%. The yield of purified oligonucleotide was 17.3 OD units.

Preparation of oligonucleotide furfuryl construct (3)

Amino functionalised oligonucleotide(1) (15.3 units) was dissolved in 700µl deionised water and 100µl buffer added (1M sodium carbonate pH 9.0). A solution of compound(2) (200µl of 10 mg/ml in DMF) was added and

the reaction allowed to proceed overnight at room temperature. The reaction mixture was then passed down a column of Sephadex G10 which was eluted with ethanol: water (1:4 vol/vol) to remove excess reagent. The oligonucleotide product(3) (13 units) was eluted in the void volume.

Conjugation reaction

The above oligonucleotide(3) (11.5 units) was dissolved in 800µl deionised water and a solution of 6-maleimidocaproic acid ((4), 200µl, 10mg/ml in ethanol) was added. The reaction was incubated overnight at room temperature, after which time low molecular weight compounds were removed from the product(5) by gel filtration as before.

Characterisation of products

Hplc analysis of the oligonucleotide species (1), (3) and (5) was carried out on an ODS Hypersil column (150 x 4.6 mm) at a flow rate of 1ml/min with detection by UV at 254nm. Gradient profile (A: 0.1 M triethylammonium acetate pH 7.0, B: acetonitrile)

| Time (min) | B(%) |
|---|---|
| 0 | 5 |
| 3.0 | 5 |
| 30.0 | 30 |

The retention times of the oligonucleotide species produced in the above reactions were well separated on this system. Their retention times are shown below.

| Oligonucleotide | Retention time (min) |
|---|---|
| (1) | 13.7 |
| (3) | 17.7 |
| (5) | 16.85 |

The molecular weights of the oligonucleotide products were confirmed by electrospray mass spectrometry as shown below.

| Oligonucleotide | MW(obs.) | MW(calc.) |
|---|---|---|
| (1) | 3209 | 3206 |
| (3) | 3418 | 3413 |
| (5) | 3629 | 3624 |

These molecular weights observed are satisfactory given the instrumental errors involved and confirm that the desired reactions had occurred.

Preparation of N-furfuryl-deoxycytidine phoshporamidite (6)

To furfurylamine (19.5ml, 200mmol) in a 500ml round bottom flask was added with stirring at 0°C a solution of sodium metabisulphite (41.8g, 220mmol) in water (160ml) over a period of 1 hour. Deoxycytidine

hydrochloride (4.3g 16.3mmol) was then added to the slightly cloudy solution. The pH of the solution was then adjusted to 7.0 - 7.1 by addition of a concentrated sodium hydroxide solution. The clear pale yellow solution was then heated to 70°C for 12 hours, after which time tlc analysis (2-propanol: ammonia: water 60:15:5) showed complete conversion of starting material rf 0.63 to a new spot at rf 0.54.
The pH of the solution was then brought to 9 by addition of a concentrated sodium hydroxide solution and the reaction was evaporated under vacuum to a yellow paste. The solid was dissolved in water (200ml) and applied to a C-18 reverse phase silica column (100g). The column was eluted with water (ca. 1500ml) until silver nitrate tests showed that no traces of chloride were present. The product was eluted by application of a gradient formed from water (1500ml) and water: methanol (1:1, 1500ml). Fractions were examined by tlc as above and those containing pure product were pooled and evaporated to yield a yellow gum. The product was then dried by acetonitrile co-evaporation (2 x 50ml) and dried under high vacuum to yield 4.62g yellowish solid product (92% yield)

This was then converted to the 5'-dimethoxytrityl derivative by reaction with dimethoxytrityl chloride in pyridine. Following on standard aqueous workup the product was purified by chromatography on silica gel using a gradient of 0-5% methanol in dichloromethane. Pure product was isolated as a pale tan foam in 65% yield.

$^1$H nmr: 200 MHz (CDCl3) 7.89 (d, 1H, H6); 7.15-7.45 (m, 11H, aromatic, furan, H5); 6.82 (d, 4H, aromatic); 6.30 (m, 3H, furan, H1'); 5.30 (br s, 1H, NH); 4.65 (br s, 1H, OH); 4.52 (m, 1H, H3'); 4.07 (m, 1H, H4'); 3.78 (s, 6H, OCH3); 3.43 (m, 2H, H5', H5''); 2.53-2.64 (m, 3H, H2', CH2-furan); 2.22 (m, 1H, H2'')

This compound was then converted into the cyanoethyl phosphoramidite derivative suitable for use on an automatic synthesiser by standard methods. The required compound (6) was isolated by flash chromatography in 80% yield as a pale foam in >98% purity as judged by hplc analysis. The satisfactory performance of this compound in the preparation of oligonucleotides was verified by determination of the stepwise coupling yield based on the intensity of the dimethoxytrityl cation released during synthesis (Gait, 1984). This coupling value was found to be 99%, indicating that its performance in synthesis compares favourably with standard unmodified nucleosides. Several oligonucleotides bearing both terminal and non-terminal modifications were synthesised and characterised by mass spectrometry. In all cases, the derived mass data agreed with the theoretical to within experimental error.

<u>Conjugation of oligonucleotide derived from (6) to alkaline phosphatase</u>

The sequence synthesised was (5') XGGGTGAATTACAAGCTCCGT (3'), where X = compound (6). Coupling yields during synthesis (as monitored by the release of dimethoxy

trityl cation) were > 98.5%. The yield of purified oligonucleotide was 15.9 OD units.

Conjugation to maleimide-activated alkaline phosphatase (Pierce Chemical Company, Rockford, IL, USA) was carried by reacting this functionalised sequence (1.84nmol, 0.36OD) with enzyme (1.12nmol) in a total volume of 75µl 1x SSC buffer. After 2 hours the conjugate was isolated in 79% yield by separation from unreacted oligonucleotide by gel filtration chromatography (Micro Bio-Spin 30 column, BioRad Laboratories, Hemel Hempsted, UK). The ratio of oligonucleotide to enzyme was estimated from the UV absorption charactersitics of the conjugate to be 0.9:1.

Preparation of compound (7)

Diaminopropane (16.5ml, 200mmol) was slowly added to a solution of sodium metabisulphite (41.8g, 220mmol) in ice cold water (160ml). Deoxycytidine hydrochloride (4.3g, 16.3mmol) was then added and the pH taken to 7 by addition of concentrated hydrochloric acid. The reaction was heated to 70°C for 8 hours. The reaction was monitored for completeness, worked up and purified as described for the preparation of compound (6), yielding the intermediate N-(3-aminopropyl)-deoxycytidine as a yellow oil.

This oil was co-evaporated with pyridine (4 x 30ml) and suspended in pyridine (20ml). Trifluoroacetic

anhydride (3ml) was then added slowly at 0°C under argon. After 2 hours tlc (25% methanol:dichloromethane) showed that all the starting material had been transformed into a material of higher rf. Methanol (20ml) was added, the reaction mixture evaporated and subjected to standard aqueous workup. The crude product was converted to the corresponding 5'-dimethoxytrityl compound by reaction with dimethoxytrityl chloride in pyridine. After aqueous workup, this material was dissolved in methanol (80ml) and concentrated ammonia (20ml) was added to remove the transient trifluoroacetyl-protecting group. After 12 hours at room temperature, the reaction mixture was evaporated to dryness to yield 5'dimethoxytrityl-N-(3-aminopropyl)-deoxycytidine (5.2g, 55% yield).

This product (8.8mmol) was dissolved in dichloromethane (25ml) and furan active ester (2) (2.74g, 8.5mmol) was added, followed by triethylamine (1.25ml, 9mmol). After 30 minutes tlc (9:1 dichloromethane: methanol, 0.5% triethylamine) showed that all starting material rf 0.0 had been converted to a new spot rf 0.38. Following on standard aqueous workup, this compound was subjected to chromatography on silica gel using a gradient of 0 - 5% methanol in dichloromethane to yield the product in >96% purity as judged by hplc (3.0g, 45% yield).

$^1$H nmr: 200 MHz (CDCl3) 8.03 (d), 7.15-7.45 (m), 6.80 (d), 6.32 (m), 6.19 (m), 5.30 (m), 4.60 (m), 4.36 (m), 4.15 (m), 3.77 (s), 3.41 (m), 2.53 (m), 2.25 (m), 1.64 (m)

This compound was then converted into the cyanoethyl phosphoramidite derivative suitable for use on an automatic synthesiser by standard methods. The required compound (7) was isolated by flash chromatography in 88% yield as a pale foam in >96% purity as judged by hplc analysis.

Conjugation of oligonucleotide derived from (7)

The sequence synthesised was (5') XATACAACACACCTTAAT (3'), where X = compound (7). Coupling yields during synthesis (as monitored by the release of dimethoxy trityl cation) were > 99.0%. The yield of purified oligonucleotide was 16 OD units. This oligonucleotide was then tested for its reactivity in the Diels Alder reaction as described for the testing of oligonucleotide (3) above. Hplc analysis showed conversion of oligonucleotide (retention time 9.45 minutes) to conjugate (retention time 8.93 minutes) cleanly over a period of 2 hours at room temperature.

The successful introduction of oligonucleotides into cells still remains a key problem in the use of genetic techniques. One possibility is to target oligonucleotides to specific cell types and to aid their subsequent transport into these cells. This would make use of the specificity and uptake properties of certain peptides such as those of viral coat proteins and other peptides and proteins some of which have been shown to have remarkable cell membrane penetrating properties. Chemically linking such

peptides and proteins to oligonucleotides provides a useful tool in allowing oligonucleotides to be easily introduced into cells. Other techniques currently under investigation for this purpose focus on modifying the oligonucleotides themselves, notably in producing oligonucleotides as prodrugs.

The most significant market area for the peptide linked oligonucleotides produced by the present invention is in the field of antisense, where targeting of very high value biologically active oligonucleotides to specific cell types could be very advantageous because of the reduced amounts of material which would have to be administered. Increasing the effectiveness of uptake and the specificity of the antisense construct for the appropriate cells could have a dramatic effect on the doses required.

A further use of oligonucleotide peptide conjugates lies in the area of labelling. The attachment of specific peptides to oligonucleotides provides a potentially limitless number of labelling tags, each recognised by an antibody specific for the peptide in question.

The markets for labelled oligonucleotides are already well established. The commonly used small molecule labels are biotin, digoxygenin, and various fluorescent molecules. Direct labelling with enzymes is less frequently used, because of the difficulty of preparation and maintenance of enzyme activity on storage. Thus there are only a limited number of

suitable markers available for oligonucleotides. The use of peptide markers would vastly increase the number of labelling species available, these being limited only by the availability of specific antibodies. Although the number of species with which these antibodies could be labelled is restricted to a limited set of (predominantly) fluorescent molecules, it could be envisaged that multiple probing experiments would be possible by sequential dissociation of hapten antibody complexes and addition of a different labelled antibody. In some senses, it could be said that the fluorescent molecule is given specificity by virtue of its conjugation to antibody and that the labelling properties so gained are reversible. Such multiple probing and re-probing techniques are valuable in reducing the number of experiments and samples which have to be processed. Current techniques for re-probing involve melting off the first nucleotide probe from the sample and then re-annealing with a second probe. The present proposal could provide advantages over this.

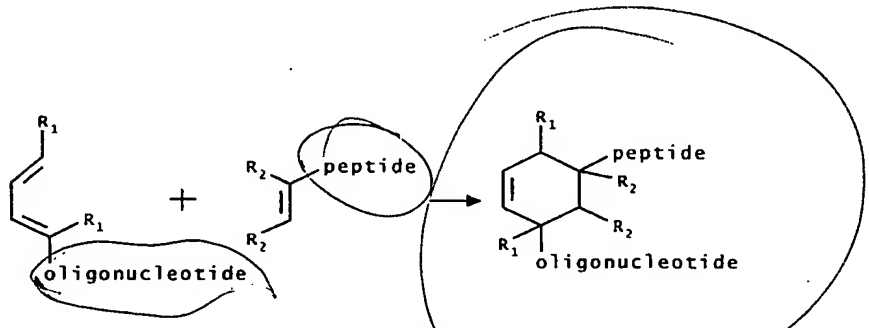
A third area of application is in the increasingly important field of linking gene function to sequence. A peptide linked to a gene fragment could act as a substrate for the product of that gene, thus allowing the linkage of gene structure with function to be made. This is an area of greatly growing importance, given the large number of sequences of unknown function being generated by sequencing projects and the drive to refine and improve the properties of known enzymes by molecular evolution and other molecular biology

techniques. Amongst the types of enzyme which could be examined using such a system are proteases, protein kinases, protein phophatases, angiotensin converting enzyme, soluble receptors and many others. There are no techniques which address this type of problem at present. Gene translation arrest techniques allow linkage of gene to gene product, but give no information about product function. The only demonstration of this type of experiment to date has a DNA sequence linked to the gene as substrate for the gene product.

This invention would be particularly useful in biotechnology research in areas of application as diverse as the production of industrial enzymes and the further understanding of molecular signalling cascade

Claims

1. A process for conjugating a peptide with an oligonucleotide, the process comprising the steps of attaching a diene to the peptide and a dienophile to the oligonucleotide, or attaching a diene to the oligonucleotide and a dienophile to the peptide, and reacting the so-formed components by means of the following reaction,




wherein R1 and R1' are electron donating groups and R2 and R2' are electron withdrawing groups, or R1 and R1' are electron withdrawing groups and R2 and R2' are electron donating groups.

2. A process as claimed in Claim 1 wherein the electron donating groups are chosen from the group comprising hydrogen, alkyl, cycloalkyl, aryl, S, O, N or heterocylic structure including these and wherein the electron donating groups may be joined to form part of a cyclic structure or they may be acyclic.

3. A process as claimed in Claim 1 or Claim 2 wherein the electron withdrawing groups are chosen from the group comprising nitro, nitrile, suphonic acid, carboxylic acid, aldehyde, carbonyl, sulphate,

sulphone, quaternary ammonium or heterocylic structures containing these wherein the electron withdrawing groups may be joined to form part of a cyclic structure or they may be acyclic.

4. A process as claimed in any of the preceding claims wherein the dienophile is attached to the peptide using an N-maleimide group wherein maleimido amino acid is incorporated during a step in the synthesis of the peptide.

5. A process as claimed in Claim 4 wherein the maleimido amino acid is incorporated in the last step of the synthesis of the peptide.

6. A process as claimed in any of the preceding claims wherein the diene is attached to the oligonucleotide wherein an amino functionalised oligonucleotide is reacted with an active ester of the diene.

7. A process is claimed in any of claims 1 to 6 wherein the diene is attached to the oligonucleotide and the diene is added during synthesis of the oligonucleotide through incorporation using a phosphoramidite monomer.

8. A process is claimed in any of the preceding claims wherein a dienophile associated peptide is linked to a diene associated oligonucleotide in a polar aqueous environment.

9. An N-furfuryl deoxycitidine phosphoramidite monomer for use in a process as claimed in any of the preceding claims.

10. An oligonucleotide linked to a diene or dienophile moiety for use in the process of conjugation of a peptide with an oligonucleotide.

11. A peptide linked to a diene or dienophile moeity, for use in the process of conjugating a peptide with an oligonucleotide.

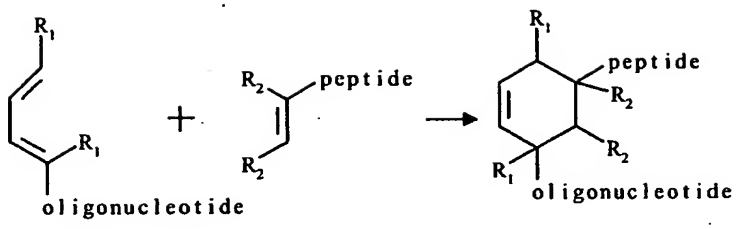



**Figure 1**: Diels Alder reaction scheme

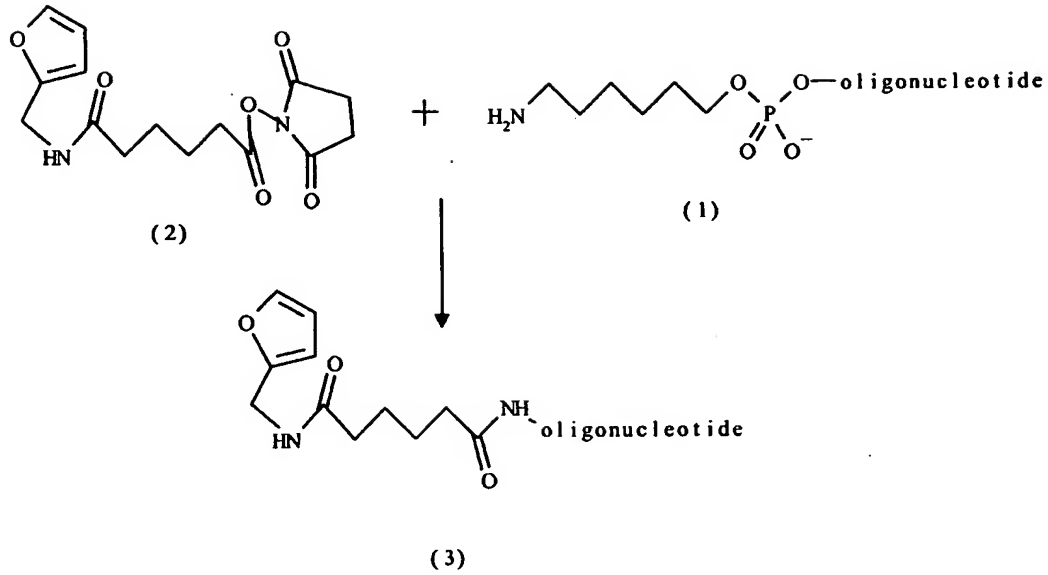



**Figure 2**: Attachment of Diene to Oligonucleotide

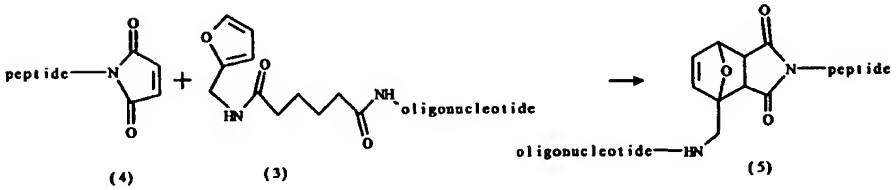
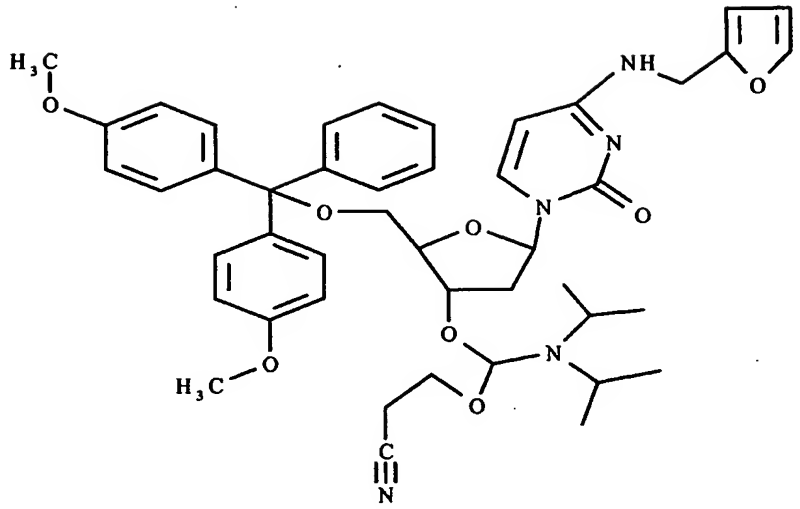
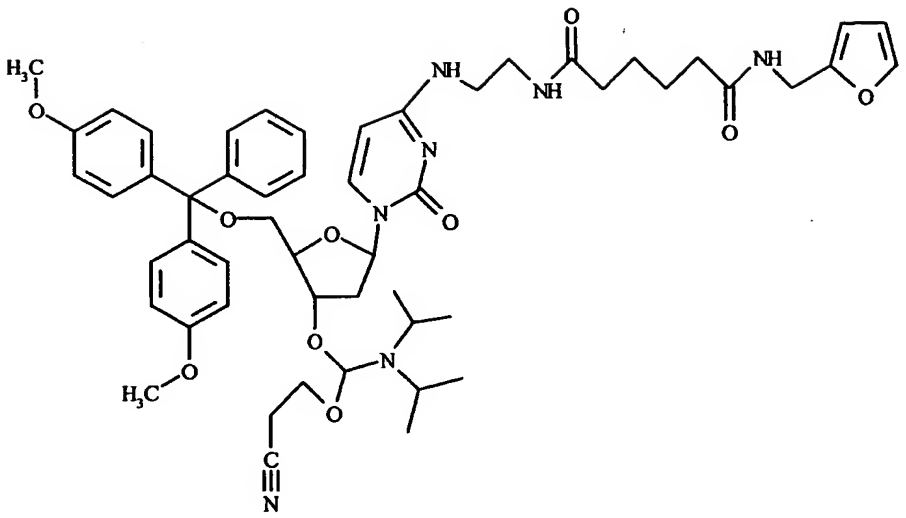



**Figure 3**: Conjugation of peptide molecule to an oligonucleotide molecule



(6)

**Figure 4**: N - furfuryl deoxycitidine phosphoramidite monomer



(7)

**Figure 5:** Phosphoramidite Monomer

# INTERNATIONAL SEARCH REPORT

Inter. xnal Application No
PCT/GB 99/03912

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 24 March 2000 | 03/04/2000 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Bardill, W |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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